The Identification of Unknown Tapeworms in Bobcats

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Abstract

Bobcats (*Lynx rufus*) in the Midwestern United States are frequently found to be hosts for several species of epizootic and zoonotic parasites, including tapeworms. Populations of bobcats are increasing, as well as the concerns that this will result in an increase in the reservoir numbers and overall range of parasites. To survey parasite loads in natural populations of bobcats, one hundred intestines were analyzed for the prevalence and identity of gastrointestinal parasites. These samples were obtained legally in March of 2012 as discarded tissue from the Department of Natural Resources Facility in Madison, WI. Wherever possible, tapeworms were identified based on proglottid and scolex morphology. Unfortunately, because the intestines were frozen and thawed multiple times before samples were collected, the morphology was often compromised. In order to definitively identify the tapeworm species from tissues sampled, our goal was to develop PCR primers that would generate specificity for the cestodes species found in Midwestern bobcats. Based on previous research studies, bobcats in this region are commonly infected with tapeworms of the genus *Diphyllobothrium* and *Taenia*, due to diet and range. Currently published genomic sequence of *Diphyllobothrium* was not sufficiently complete to allow identification of primers, however, primers were developed from the entire range of available published sequence of *Taenia solium* (pork tapeworm). Successfully amplifying primers will be used to compare the genomic DNA sequence from these bobcat tapeworms to museum samples previously identified through expert morphological assessment to serve as comparative controls for species identification. Currently, cestode DNA fragments amplified from several samples are being prepared for sequencing. Mitochondrial sequencing results will be used to confirm species identification wherever possible. This research is a continuing project and is complicated by the fact that none of these tapeworm species have a completely sequenced genome.

Background

Parasites, specifically tapeworms, are capable of infecting and residing within domestic and wild animals. One of the most abundant species throughout North America infected by these parasites are bobcats (*Lynx rufus*). Currently, bobcat populations are increasing in nearly every state in the U.S. depending on the habitats. Different communities of parasites tend to infect the animals depending on their geographical range. Parasitic communities correlate with the climate, prey availability, and the habitat of the region. Many of the parasites that infect the bobcats are also capable of infecting other carnivores such as coyotes, cougars, domestic cats, and domestic dogs. According to a previous study on bobcats residing in the Midwest, specifically Illinois, *Diphyllobothrium latum* was the most common tapeworm infecting the animals and resulting in zoonotic disease. Other species that were found in high prevalence within the bobcats included *Taenia rileyi*, *Alaria marcianae*, and *Toxocara cati*. According to another study which focused on helminth species diversity and biology in the bobcat specifically within the state of Nebraska, the species *Taenia rileyi*, *Taenia pisiformis*, and *Taenia macrocystis* were some of the highest in prevalence for the cestoda found. The bobcats studied for this research project originally resided in the state of Wisconsin. Bobcats are mainly found in Northern Wisconsin, while some are found in Central and Southern Wisconsin. Due to their known diet and previous studies done on cestodes within bobcats that reside in the Midwest United States, the parasites usually included species of tapeworms from the *Taenia* and *Diphyllobothrium* genus. *Diphyllobothrium spp*. are commonly found within bobcat intestines and generally use fish, amphibians, or reptiles as their intermediate host and the bobcat as their definitive host. *Taenia* is also known to utilize bobcats as their definitive host, with rodents as their intermediate host. The goal of this study was to determine the species of tapeworms found in Wisconsin bobcats using molecular techniques.

Methods

- Bobcat intestines were collected from legally harvested animals in March of 2012 from the DNR trapping examination station in Madison, Wisconsin. Intestines were frozen and labeled until needed for further study. To isolate tapeworm specimens, intestines were cut open and the fecal matter was flushed with running water into sieves. In addition, the small intestines were bisected and examined for tapeworms. When a tapeworm was found, it was carefully removed and preserved in 70% ethanol.

- DNA extraction was performed following established protocols using the Qiagen DNeasy Tissue Kit. Primers were designed using Primer-BLAST and Primer-3 on the published *Taenia solium* complete scaffold region (122 Mb). Nine primer pairs were identified (Table 1) with one pair selected from different regions to distribute the selections over the length of the ~450 Mb of completely sequenced genome.

Table 1: Custom Primers Designed from the Published *Taenia solium* sequence*

Primer Pair	Forward Start Base Location	Reverse End Base Location	Expected Fragment Size
1	24168	24989	822
2	83986	84627	642
3	140152	140545	394
4	151489	152368	880
5	244284	244756	473
6	250870	251387	518
7	310376	311098	723
8	371545	372033	489
9	402114	402733	620

*Actual linear contig sequence published is ~425kb of known sequence, from a genome estimated to be 120Mb in size.

•The conditions of the PCR were consistently performed using 1 uL of a concentration of 25-40 ng of DNA per reaction. ThermoFisher Scientific's DreamTaq PCR Master Mix (2X) was utilized as the master mix and diluted to 1X. Custom primers described in Table 1, along with demonstrated appropriate controls, were tested for the isolated cestode samples (1 uL of 100 uM of each forward and reverse primer per 25 uL reaction).

•The thermocycler conditions were as follows: 95 degrees for 5 minutes, 30 cycles of 95 degrees for 30 seconds, 45 degrees for 1 min, and 72 degrees for 1 minute. The final extension consisted of 72 degrees for 5 minutes, and a final hold of 4 degrees. Low stringency conditions were utilized to improve the chances of obtaining amplification products. PCR results were visualized using gel electrophoresis (1% TAE) for 45 min at 125 mV. The positive control primers (JB11 and JB12) amplify tapeworm mitochondrial DNA (*nad1* gene) and produce a specific 460-525 bp band from parasite species but not from host mammals. The negative controls included both water and bobcat DNA. Morphologically identified known specimens were obtained from the Manter Laboratory of Parasitology (Lincoln, Nebraska), and treated to the same DNA extraction and PCR protocols to act as controls.

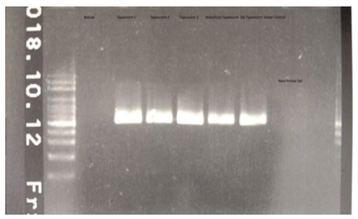
Results/Discussion

- Commonly, the small scolex region of the worms could not be isolated or had been used in prior identification studies, and in many cases only the proglottids were obtained. One scolex was successfully removed and photographed (Figure 1) and used in its entirety for DNA isolation.

Figure 1: Scolex of Tapeworm

- Gel electrophoresis was performed using primers JB11 and JB12 which amplified the *nad1* mitochondrial NADH dehydrogenase subunit 1 (Figure 2), but did not amplify any fragment from bobcat, eliminating the background contamination seen in previous studies. Lane 2, containing the bobcat intestine, successfully resulted in no bands, proving that background contamination is not present when using primers JB11 and JB12. Lanes 3-7, all containing tapeworm, resulted in band sizes of 500 bp.

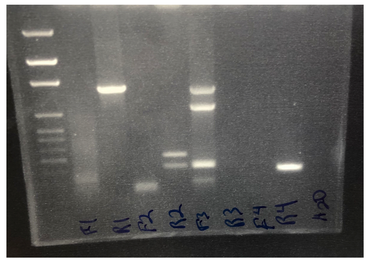
Figure 2: Mitochondrial DNA Gel Results Using Tapeworm Specific Primers JB11 and JB12 on DNA Isolated from Bobcat and Individual Tapeworms



Gel results shown in Figure 2 possess the following lane content and band size: Lane 1 contained the 100 bp ladder. Lane 2 contained Bobcat Intestine resulting in no bands. Lane 3 contained Tapeworm 1 from bobcat and resulted in a band size of ~500 bp. Lane 4 contained Tapeworm 2 from bobcat and resulted in a band size of ~500 bp. Lane 5 contained Tapeworm 3 from bobcat and resulted in a band size of ~500 bp. Lane 6 contained Waterford Tapeworm *D. latum* from a human and resulted in band size of ~500 bp. Lane 7 contained Tapeworm 4 from bobcat and resulted in a band size of ~500 bp.

- Figure 3 shows the gel using novel primers developed from the *T. solium* scaffold (listed in Table 1). These primer pairs failed to produce expected bands. Primers were tested individually on the isolated tapeworm sample (Figure 1) using 99 cycles of PCR to determine if any extension occurred. Forward Primers 1, 2, 3, and 4, as well as Reverse Primers 2 and 3 resulted in multi-band patterns or no bands. Reverse Primer 1 and Reverse Primer 4 resulted in single bands. These bands, which resulted from the priming of a single primer only, were repeatable, and these fragments are being isolated for sequencing. Sequencing data obtained from these fragments will be compared to the published sequenced data to attempt to definitively identify species, and determine which primers may be useful for further studies.

Figure 3: Gel Results Using Novel Primers Developed from *Taenia solium* Scaffold on DNA Isolated from Bobcat Tapeworm 4



Gel results using novel primers on Bobcat Tapeworm 4 shown in Figure 3 possess the following lane content and band size: Lane 1 contained the 100 bp ladder. Lane 2 contained Forward Primer 1 and resulted in a smear with no specific bands shown. Lane 3 contained Reverse Primer 1 and resulted in a single band of ~2K bp. Lane 4 contained Forward Primer 2 and resulted in a band at ~250 bp. Lane 5 contained Reverse Primer 2 and resulted in bands at ~500 bp and ~400 bp. Lane 6 contained Forward Primer 3 and resulted in bands at ~1.7K bp, ~1.3K bp, ~400 bp, and ~300 bp. Lane 7 contained Reverse Primer 3 and resulted in no bands. Lane 8 contained Forward Primer 4 and resulted in no bands. Lane 9 contained Reverse Primer 4 and resulted in a single band at ~350 bp. Lane 10 contained H2O and resulted in no bands.

Conclusion

Currently, cestode DNA fragments amplified from several samples are being prepared for sequencing. Complications occurred due to the lack of published data to derive primers from and compare results. PCR primers to amplify the ITS1 and ITS2 regions of 28s eukaryotic ribosomal subunit will be tried next, because it is a well characterized region with published sequences for different species of cestodes. This is a continuing research project with the overall goal of being able to uniquely identify species based on a PCR profile.

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